Application of Gene Expression Analysis with Microarrays and Proteomics to the Problem of Hemorrhagic Shock and Resuscitation[1]

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ABSTRACT

Hemorrhage is the principal cause of death of soldiers on the battlefield. With dispersed troops and future combat operations expecting longer evacuation times and limited availability of medical supplies far-forward, significant improvements in fluid resuscitation will be required if casualties are to be saved. While it is known that a drop in blood pressure below 40 mm Hg or loss of more than 50% of the blood volume is fatal, most cells in the body, with the exception of brain cells, can survive for several hours with minimal oxygen or nutrients. Hence, morbidity from blood loss involves factors beside lack of oxygen and nutrients. Little more is known, however, about how the body responds to loss of blood or which organs are most affected. An understanding of the temporal responses of tissues to hemorrhage will lead to improved strategies of intervention before irreversible deterioration occurs.

We are using gene expression analysis with microarrays to assess the responses of various organs to severe hemorrhage in rodents to uncover the prominent metabolic pathways involved. Until recently, traditional molecular techniques allowed analysis of only one gene at a time. Throughput was very limited and an accurate picture of the molecules that orchestrate the regulation of health and the dysfunction that occurs during disease or injury has not been possible. The microarray, which allows analysis of changes in expression of thousands of genes, promises to help clarify the molecular and genetic basis of health and disease and speed drug discovery. This information will guide the rational development of new resuscitation fluids with appropriate drug additives.

1.0 BACKGROUND

Although hemorrhage is the principal cause of death of soldiers on the battlefield and is an important component of injury in civilian trauma, we possess only a rudimentary understanding of the cellular basis for the physiological alterations that occur following severe blood loss in any mammalian species. Consequently, designing therapies to meet cellular needs following the global ischemia of severe hemorrhage has been primarily through trial and error. Utilizing recent advances in genomics and proteomics, the present endeavor attempts to better understand the response of the mammalian organism to hemorrhage and to begin to

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systematically investigate cellular responses to the global ischemia that occurs during severe blood loss in order to provide new strategies for saving the lives of trauma patients who will otherwise die from their wounds.

1.1 Microarrays

Although each cell in the body contains a complete set of instructions (the genome) for specifying all the functions of the body, only a limited amount of this genetic material is active, and the portions of the genome that are active are specific for each cell type. The repertoire of the thousands of genes that are expressed in each cell type is termed the transcriptome. Until recently, traditional molecular techniques allowed analysis of only one gene at a time. Microarrays, so-called because many thousands of fragments of genes can be packed into an area of several square centimeters, are also known as DNA chips or gene chips, and they represent the first widely used application that builds upon the information provided by sequencing genome projects to the study of biological questions.

Knowledge about DNA sequences allows definition of genes by a unique, relatively small piece of the gene. The technique for making gene chips by synthesizing short oligonucleotides onto a glass substrate by a photolithographic process, was first introduced by the biotechnology company Affymetrix (Santa Clara, CA) [2, 3]. Most laboratories produce their own chips by spotting preformed complementary DNA (cDNA) or oligonucleotides by a technique developed by Patrick Brown's laboratory at Stanford University [4-6]. During the course of a study, one can collect samples of blood or tissues at various times. Then the RNA from each sample is isolated and a copy made with an enzyme that can generate cDNA. This cDNA is combined with the complement attached to the chip and after removal of unbound material, it is scanned by a fluorescent scanner to detect sites of molecular hybridization to determine if that gene was being expressed by the cell or tissue under investigation at the time the messenger RNA (mRNA) was isolated. The chemical conditions necessary for allowing this specific, one-to-one combination, known as molecular hybridization, are very well defined. This application of microarrays is termed gene expression analysis. If one wants to know the affect of a drug or disease on the activity of many genes, gene expression analysis is one of the least expensive and most robust techniques currently available. By combining this technology with computers that can track and record the activity of genes, thousands can be followed simultaneously.

1.1.1 Subarrays

Figure 1 shows a subarray of 480 nucleic acid fragments produced at the US Army Institute of Surgical Research. Each spot is about 120 microns (0.12 mm) in diameter, and was deposited by a stainless pin in a special computer-controlled robot. The exact order of each spot is tracked by appropriate software on the computer. About 80,000 spots can be produced on a standard microscope slide. Although there is still controversy about the exact number of genes in mammalian genomes, (estimates are currently about 40,000), representative fragments of the entire genome can in theory be placed on microarrays and all genes analysed simultaneously. RNAs, the immediate products of genes, are the effectors of the transcriptome. The RNAs are isolated and complementary copies (cDNA) made that incorporate a fluorescent dye. When hybridized to the array, each cDNA finds its appropriate complementary sequence on the array, roughly in proportion to its concentration in the cell. By quantifying the fluorescence in a laser-activated scanner, the quantity of RNA present in the original mixture can be determined. In practice, an appropriate control from organs or cells that have not been perturbed is labeled with one color fluorescent dye while the experimental sample is labeled with a different colored dye. The figure below shows an example of a subarray from a microarray used for quantifying gene expression from rat tissues.

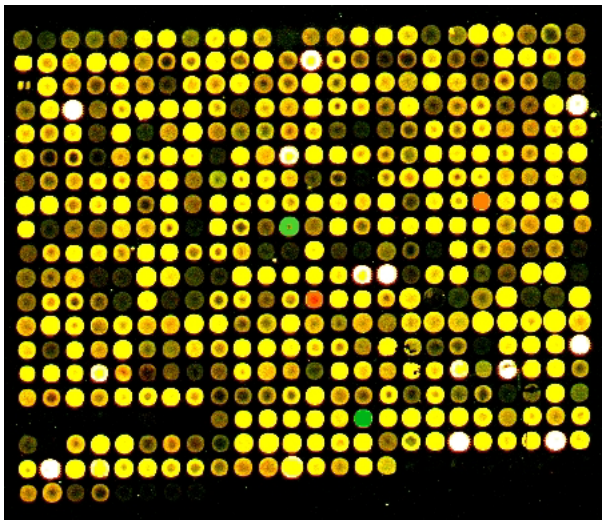

Figure 1: A subarray containing 480 oligonucleotides for interrogating 480 genes for alterations in gene expression. Yellow = no change in expression, Green = downward expression and Red = upward regulation of gene expression.

2.0 MICROARRAY APPLICATION

One example of the use of microarray technology applied to the problem of hemorrhagic shock and resuscitation at the US Army Institute of Surgical Research is examining the genetic responses to 40% hemorrhage in rat and mouse models as a function of time (1, 3, 6, 12, 24, and 48 hours). Analysis of these results in the lung indicates that biochemical pathways for biogenic amines, eiconosoids, inflammation, and steroid metabolism were prominently affected ($p<0.05$). By performing similar analysis in other organs (liver, kidney, and intestine) and following up these results with proteomic analysis, it is hypothesized that a set of common metabolic pathways will be identified and confirmed that is affected by severe blood loss. This information will guide the rational development of new resuscitation fluids with appropriate drug additives. While we plan to examine several organs and tissues in these animals we are focusing first on lung as it is the predominant organ to fail in humans after severe trauma [7]. In animal models, hemorrhage produces lung injury despite locations of the primary insult elsewhere [8-11].

2.1 Analysis of Relative Gene Expression with Two Color Microarrays

Figure 2 illustrates the steps in performing gene expression analysis with microarrays. Tissues from animal intestine, liver, lung, kidney, spleen, heart, skeletal muscle (gastrocnemius) skin, and brain were removed and placed in RNALater™ (Ambion, Austin, TX): Total RNA was isolated from each tissue from each animal and its quality analyzed by gel electrophoresis. A reference preparation consisting of equal amounts of RNA pooled from 10 organs (liver, lung, kidney, spleen, heart, skeletal muscle, skin, jejunum, and brain) of untreated control animals was used as reference RNA. Five-µg samples exhibiting undegraded RNA from each rat lung were reverse-transcribed in the presence of a C-6 amine modified random hexamer and aminoallodeoxyuridine to produce fluorescent labeled cDNA. Following reverse transcription, RNA was degraded. After separation from unbound dye, the samples were again lyophilized and then reconstituted in

hybridization solution. Following hybridization on a microarray (one per rat) and washing to remove unhybridized cDNA and scanning with an Axon 4000B (Axon Instruments, Union City, CA) at 10 micron resolution, the resulting 16-bit TIFF images were analyzed with GenePix 4.1 software (Axon Instruments) for calculation of Cy5 and Cy3 fluorescence intensities at each spot.

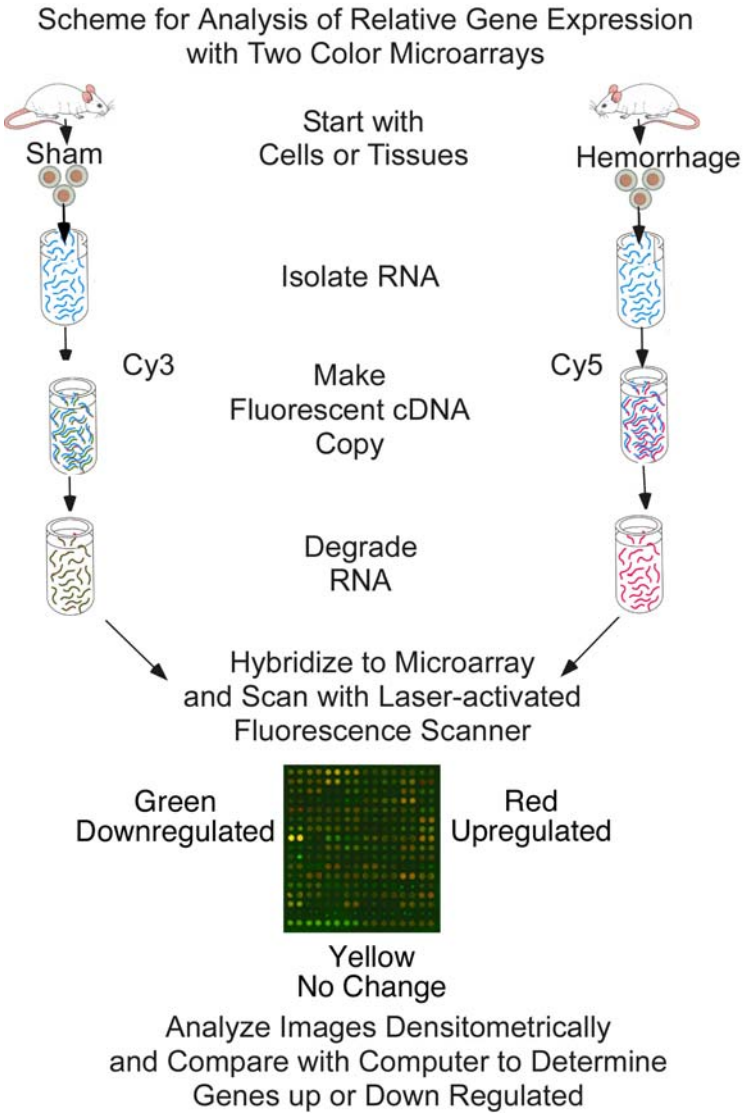

Figure 2

2.1 Cluster Analysis

The tools for handling the large data sets generated by microarray technology are in development and constantly improving and as are statistical tools. The principal tool in use currently is known as cluster analysis, which organizes data on the basis of similar patterns of expression. Figure 3 illustrates the results of alterations in gene expression in the lung of mice after a 40% reduction in blood volume as a function of time. The cluster analysis program associated genes whose expression was altered together as a function of time after hemorrhage. By performing gene expression analysis with groups of at least 3 animals, each with its own microarray, animal variation in gene expression can be ascertained. The clustered genes seen in Figure 3 all were analyzed by ANOVA and were significant at the $p < 0.01$ account for 1,146 genes out of 17,249 spotted on the microarray. Cluster analysis shows that most of the genes that were altered up scored as upregulated. Many of the genes altered in expression were upregulated at multiple times.

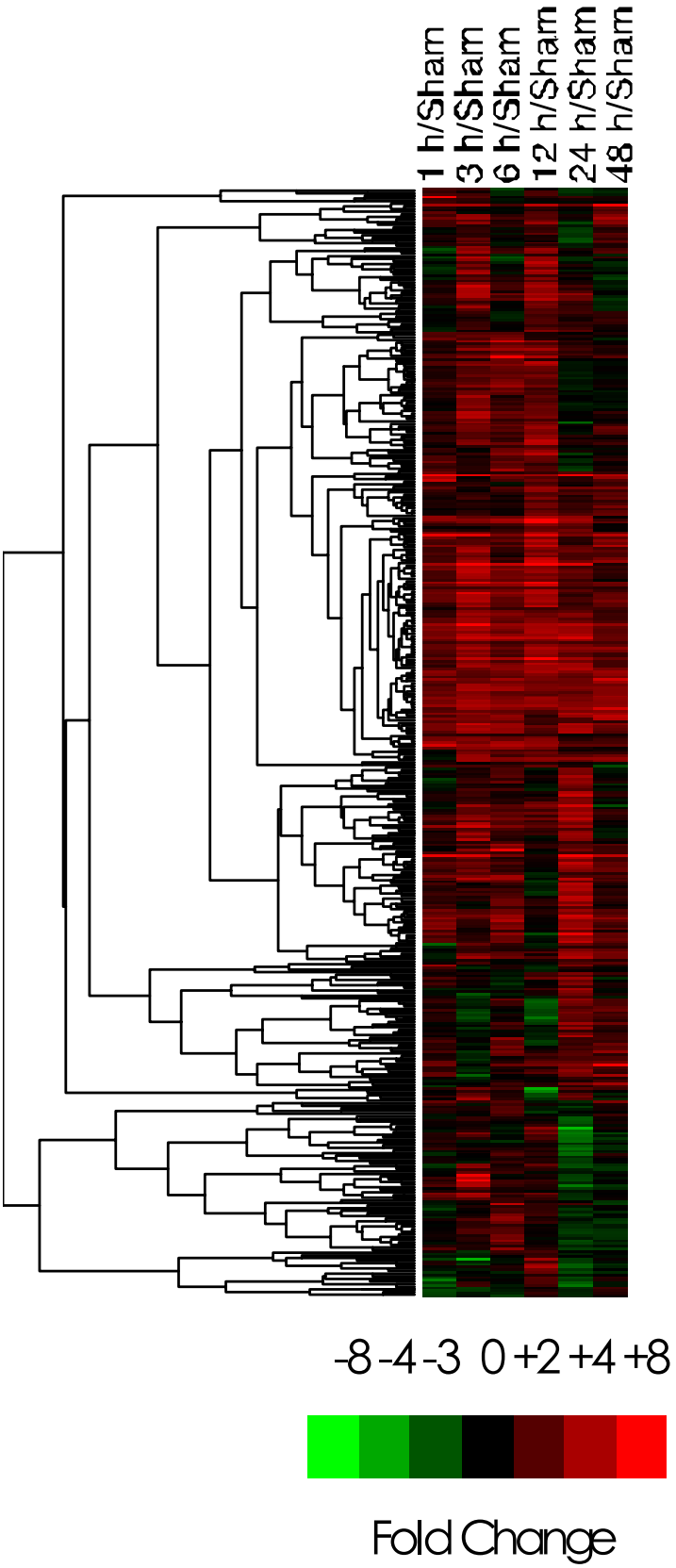
1 h/Sham
3 h/Sham
6 h/Sham
12 h/Sham
24 h/Sham
48 h/Sham
-8 -4 -3 0 +2 +4 +8
Fold Change

Figure 3

Figure 4 shows and inset of genes upregulated in Figure 3. Many of the genes altered in mouse lung are termed expressed sequence tags ESTs. It is highly likely that these are genes but the specific functions are as yet unknown. By combining these results with functional genomics and proteomics we expect to be able to determine the role of the genes in the global response to ischemia that occurs following hemorrhage. The goal of using gene expression analysis in developing resuscitation fluids will be to use this genetic information to determine if a particular resuscitation fluid is either reducing the shock response or accelerating the early return to the preshock state.

Figure 4

3.0 SUMMARY

By using microarray technology applied to the study of the genetic response to hemorrhagic shock and resuscitation in animal model organs we hope to better understand the cellular response of tissue to hemorrhage. This information will guide the development of new resuscitation fluids. We predict that as we develop better resuscitation fluids, we will see decreases in the magnitude of the genetic responses of organs to hemorrhage that will translate into an increase in survival and a reduction in the time required to recover from hemorrhage.

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